ON THE CAPACITY FOR FERTILIZATION AFTER THE INITIATION OF DEVELOPMENT.[1]

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I. An Attempt to Fertilize Sea-urchin Eggs Subsequent to Hypertonic Parthenogenesis.

Contents.

I. Introduction.

The purpose of the present paper is to present a part of the results of a study of the capacity for fertilization of eggs of the sea-urchin (*Arbacia punctulata*) after the initiation of development by artificial agents. A previous paper[2] by the writer has dealt with some of the phenomena encountered in an attempt to fertilize the eggs of *Arbacia* after a treatment with certain artificial stimuli, *e. g.*, butyric acid and heat. The ideas involved resolve themselves into the question—can eggs that have been activated by artificial parthenogenetic agents, be fertilized by spermatozoa of the same species; or does activation by hypertonic sea-water preclude fertilization as in the case of sperm activation?

[1] From the Marine Biological Laboratory, Wood's Hole, Mass., and the Hull Zoölogical Laboratory, University of Chicago.

[2] Moore, '16.

The egg of the sea-urchin cannot be fertilized before maturation has taken place, even though the spermatozoön may enter the egg. Penetration is not fertilization. However, if a spermatozoön enters immediately after maturation, normal changes characteristic of fertilization are produced. But shortly after the act of fertilization the egg returns again to a condition in which fertilization is impossible. E. B. Wilson[1] has demonstrated for *Cerebratulus* that if pieces of fertilized eggs are dropped into sea-water containing active spermatozoa there is no reaction between the pieces of eggs and sperm; however, with pieces of unfertilized eggs there is a reaction. Delage, too, has noted that starfish eggs which had been exposed to artificial parthenogenetic agents could not be fertilized.[2]

Maturation, then, leads to changes within the egg that make possible initiation of development by either a spermatozoön or an artificial stimulus. And if this initiation of development is brought about, carrying with it of course a change of the egg system, fertilization is thereby rendered impossible.

It would seem strange not to suppose that an egg passes through essentially similar physiological changes during the development from one cell to a swimming pluteus, whether activation has been produced artificially or by a spermatozoön. If activation by a spermatozoön produces a physiological state of reactivity that leads to development, and, if activation by artificial agents also produces physiological changes that lead to the same kind of development, should we not suppose that the egg—the only common factor—would pass through essentially similar conditions in the two cases? One would judge from the papers of Loeb, that this supposition is erroneous, that sperm will react with artificially activated eggs in an entirely normal manner, producing normal swimming larvæ, while it is positively known that such is not the case when activation has been previously affected by means of a spermatozoön.

The writer has presented proof that eggs of the sea-urchin activated by butyric acid, and resulting in the production of normal membranes, will not react with sperm nor become fer-

[1] Wilson, '03.

[2] Delage, '01.

tilized, as Loeb has believed,[1] but that they are entirely unresponsive to sperm, even though spermatozoa pass into the egg substance. The present paper deals with an attempted analysis of the conditions that are produced, after activation by hypertonic sea-water, with reference to the capacity of the egg for fertilization.

Loeb[2] determined that an exposure to hypertonic sea-water (50 c.c. sea-water + 8 c.c. $2\frac{1}{2}$ m. NaCl) for a certain period of time would cause sea-urchin eggs, after their return to normal sea-water, to segment in quite a normal manner: they would also undergo further developmental changes and become essentially normal plutei. But certain eggs from the culture will not have had a sufficiently optimum exposure to bring out their full developmental tendencies and will as a result of this remain quiescent after dividing into two, four or eight cells Can these eggs activated by hypertonic sea-water yet be fertilized by a spermatozoön? Loeb says they may be and uses the assertion as a proof that the egg has not been changed in its power of normal physiological reactivity; and that it may be fertilized if only a spermatozoön reaches the cortex of the egg. His hypothetical "lysin-like" substance contributed by the spermatozoön can still assert itself by producing cytolysis and his "corrective agent" of the spermatozoön may also act to check the imposed cytolytic condition and allow the egg to go on to development.

If the conditions are as Loeb believes, we will naturally have to assume that activation of the egg—the initiation of development—is accompanied in the two instances: (1) in sperm activation and (2) in activation by artificial parthenogenesis, by a different series of reactions that lead to the same end, namely the production of swimming larvæ. But if this is so we will have to assume a lack of harmony in the development of an egg when activated by different agents. In the vast number of cases in which development may be artificially initiated there is no common factor but the egg itself. Development is a series of reactions on the part of the egg whether initiation is given by a

[1] Loeb, '13.

[2] "Art. Par. and Fert.," pp. 237–238.

spermatozoön or by an artificial agent and, unless we assume such a relatively restricted set of reactions, we have as yet no explanation to offer for parthenogenesis. The principal issue at hand is, can an egg of *Arbacia* that has been once activated by hypertonic sea-water be fertilized or reactivated by a spermatozoön of the same species?

The results obtained from this study show conclusively that a treatment of the sea-urchin egg with hypertonic sea-water for an optimum length of time—*e. g.*, that in which one obtains the highest percentage of development, both in regard to cleavage and to larval production—leads to changes in the physiological state of the egg that result in the loss of its capacity for fertilization. Spermatozoa readily enter the eggs but they are unable to cause a reactivation of the egg; they appear only as inert bodies within the cytoplasm. Not only does the spermatozoön fail to produce activation changes but also the initial changes, produced by the hypertonic treatment, have created an environment in which a spermatozoön very quickly goes to pieces.

If an egg has not received the optimum exposure for activation, from the hypertonic treatment, a spermatozoön is capable to some extent of provoking activation changes, and a very striking gradient of activity on the part of the egg can be demonstrated from the normal behavior in fertilization to a complete state of responsive inactivity. If the previous treatment has led to segmentation, spermatozoa readily enter the blastomeres, but are entirely incapable of reactivating them.

And finally, the capacity for fertilization has been found to correspond with the presence or absence of the egg secretion, fertilizin. If the egg possesses any capacity for fertilization after the hypertonic treatment, fertilizin has been found to be present; when the capacity for fertilization is negative, no fertilizin has ever been detected by its sperm agglutinating properties.

II. Material and Methods.

The material used in this investigation has been confined to the Atlantic sea-urchin (*Arbacia punctulata*) found in the Woods Hole region. Fresh material was received daily from the labor-

atory live-car and was kept in running sea-water in the laboratory until used. Eggs and sperm were secured in the usual way—by cutting away the leathery oral disk and collecting the eggs and sperm in separate syracuse dishes as it is shed by the animal. Eggs were always washed in sea-water from two to four times before being used in an experiment, as this insures a more consistent lot of fertilizations than with unwashed eggs.

The method for testing the capacity for fertilization of eggs that have been activated by a hypertonic solution is the same as that employed by Loeb[1] in his studies of this nature. Eggs after washing were transferred to the hypertonic sea-water and allowed to remain for definite periods of time: at the termination of the exposure they were removed with a pipette to two separate dishes of sea-water (150 c.c.–200 c.c.) A and B. Lot A was allowed to stand at room temperature without further treatment, while to lot B was added a quantity of a fresh sperm suspension. For each experiment both a fertilized and an unfertilized lot were set aside as controls.

III. Observations of Living Material.

Since there is a great difference in the effects of different concentrations of, and variable time exposures to, hypertonic sea-water the experiments with the sea-urchin egg have included quite a range of concentrations and periods of exposure, but to simplify the data, only results from two concentrations of the hypertonic solution will be reported at this time. The two concentrations may then be known as the weaker solution (50 c.c. sea-water + 8 c.c. $2\frac{1}{2}$ m. NaCl) and the stronger solution (50 c.c. sea-water + 16 c.c. $2\frac{1}{2}$ m. NaCl).

These two concentrations of hypertonic sea-water were employed because of the very different results obtained with them. An exposure to the weaker solution results in the segmentation of a relatively few eggs (see Table I. and Fig. 1, *a*) but usually a very small percentage of the eggs will segment once or twice and cease their development, while a similar exposure to the stronger solution gives a much higher percentage of cleavages and a much greater number of swimming larvæ. An exposure

[1] "Art. Par. and Fert.," pp. 235–236.

to the latter solution is much more nearly an optimum treatment for activation than is the weaker concentration.

1. *Fertilization after Exposure to Hypertonic Sea-water.*

The fundamental interest of the writer has been directed towards the capacity of an egg, once activated by an artificial stimulus, for fertilization. If an egg is fertilized normally, the spermatozoön supplies the optimum stimulus for the initiation of changes, latent within it, that lead to normal development. But since these same changes may be initiated (it seems that we must regard it so) by artificial agents, is there yet the possibility of an observable stimulus from a spermatozoön if applied subsequent to or during these changes? Can fertilization be superimposed upon hypertonic parthenogenesis?

Eggs were exposed to the action of hypertonic sea-water as given above, returned to normal sea-water and divided into two lots, one of which remained standing at room temperature, while to the other was added a fresh sperm suspension. Let it again be brought to mind that exceedingly variable results are obtained from the same type of treatment of two different lots of eggs and since the condition of the eggs before sperm are added is the all-important factor for subsequent fertilization one must be very careful to know as thoroughly as possible the condition of the eggs in question. In one experiment one may obtain only 3 per cent. of cleavages after a given hypertonic treatment and at another time, under as nearly as possible identical conditions, 25 per cent. to 35 per cent. cleavages. The following experiment will indicate the method of procedure and the results in general that have been obtained.

From the results of this experiment one can readily see the different capacities for fertilization possessed by eggs exposed to the weaker solution (lot A) and those activated by the stronger one (lot B). This relation can be seen at a glance from Fig. 1, the curves of which were plotted from this experiment.

The lot of eggs returned to normal sea-water and inseminated, after an exposure to the weaker solution A, for a period of ten to twenty minutes, appear very little, if any, different from the control lot of normally inseminated eggs, either as regards

Experiment:

4:30 P.M. Eggs collected and washed. Aug. 26, 1916.

5:00 P.M. Divided into two lots *A* and *B*.

A, placed in (50 c.c. sea-water + 8 c.c. $2\frac{1}{2}$ m. NaCl).

B, placed in (50 c.c. sea-water + 16 c.c. $2\frac{1}{2}$ m. NaCl).

Eggs were returned to sea-water from the two solutions by means of a pipette and divided into two lots one of which remained without further treatment (control): to the other was added immediately a fresh sperm suspension.

TABLE I.

Lot A.

No.	Returned to Sea-water.	Hypertonic Solution Alone. Percentages of		Hypertonic Solution Plus Sperm. Percentages of	
		Cleavages.	Swimming Larvæ.	Cleavages.	Swimming Larvæ.
1	5:10 P.M.	0	0	99	90
2	5:20 "	0	0	95	85
3	5:35 "	1	0	83	70
4	5:50 "	2	0	70	55
5	6:00 "	3	1	60	35
6	6:20 "	2	0	28	20
7	6:35 "	2	2	33	20
8	6:50 "	4	0	30	15
9	7:05 "	5	1	28	5
10	7:20 "	4	1	20	15
11	7:35 "	6	0	35	10
12	7:50 "	8	0	35	5

Lot B.

1	5:05 P.M.	1	0	22	25
2	5:10 "	0	0	18	18
3	5:20 "	10	7	12	20
4	5:30 "	22	20	20	28
5	5:40 "	18	7	18	5
6	5:50 "	14	5	9	2
7	6:00 "	12	1	10	1
8	6:20 "	5	0	1	0
9	6:35 "	6	0	6	0
10	6:50 "	4	0	1	0
11	7:05 "	2	0	2	0

the number of cleavages or the number of swimming larvæ. But since an exposure of this intensity is practically ineffective in producing initiation of development, is it not to be supposed that the eggs can yet be fertilized by sperm? They are, so far as we may judge, entirely normal or essentially so. However, as the length of exposure to the hypertonic solution is prolonged the capacity for fertilization drops off until after an exposure of two hours' duration scarcely any larvæ appear as a result

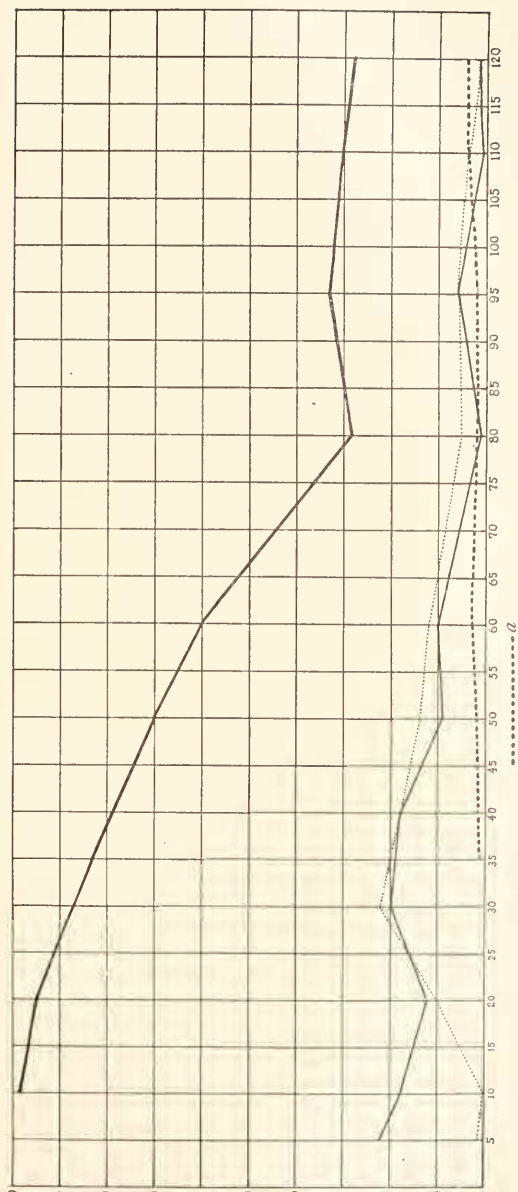

FIG. 1. Curves showing the initiation of development of *Arbacia* eggs as measured by cleavages, after an exposure to hypertonic sea-water; and the relative capacity for fertilization after an exposure to the different concentrations. *a*, weaker hypertonic solution alone; *a'*, weaker hypertonic solution followed by insemination; *b*, stronger hypertonic solution alone; *b'*, stronger hypertonic solution followed by insemination. The ordinates represent percentages of cleavage; and the abscissæ, time of exposure in minutes.

of insemination.[1] This is undoubtedly due to the condition of the egg, produced as a result of the hypertonic exposure· its capacity for fertilization is being gradually decreased.

We may correlate, it seems, this gradually progressing unfertilizable condition of the egg that appears as the eggs are allowed to remain in the solution, and the escape of substances from the eggs which also increases as the eggs are allowed to remain for longer times in the solution. One can readily notice an almost entire absence of pigment from many of these eggs while others will show a decided loss of it. Probably also many other substances escape at the same time from the egg, some of which may be absolutely necessary for certain chemical combinations taking place during fertilization and development.

The type of swimming larvæ obtained in such a series of experiments shows also a very beautiful gradient in normality as well as in number. Most of those obtained as a result of fertilization, after a short hypertonic treatment, appear entirely normal, but the percentage of abnormalities increases as the length of exposure to the hypertonic solution before fertilization was lengthened: after an exposure of $1\frac{1}{2}$ hours to 2 hours practically no normal larvæ are obtained and the mortality is very high.

For the higher concentration the curve (b and b^1, Fig. 1) is decidedly different from (a and a^1), the weaker one, but yet it is quite characteristic for exposures to such a concentration. The greatest percentage of cleavages from the hypertonic solution alone are obtained in lots, the exposure of which had been thirty minutes or forty minutes in duration while often a small amount of segmentation is induced by an exposure of $1\frac{1}{2}$ hours. But distinctly different from the curve of cleavages when the lower concentration was used is the fact that the curve of fertilization (?) follows more nearly that of the hypertonic treatment alone. This is especially true when the percentage of swimming larvæ is considered. In dishes of inseminated eggs, previously exposed to the stronger hypertonic solution for 10,

[1] The number of cleavages after a two-hour exposure in this experiment is exceptionally high as compared with scores of other experiments for the same length of time. Usually such an exposure is followed by not more than 3 per cent. of swimming larvæ but many times swimming forms do not appear at all.

20, 30 or 40 minutes, quite a goodly percentage of larval forms are to be seen, but compared with the curve of larval production of the hypertonic solution alone it has been increased very little or not at all. We cannot, therefore, refer the swimming larvæ appearing in these inseminated cultures to fertilized eggs but they can be regarded only as larvæ that have developed from the action of the hypertonic solution upon the eggs. Insemination did not increase the number of cleavages or swimming larvæ over that obtained after hypertonic activation alone.

We can now consider more in detail the results of the experiment under consideration and let it serve to illustrate the results of most of the experiments.

In an exposure to the weaker concentration of hypertonic sea-water very few eggs give evidence of a cortical change that in any way resembles the fertilization membrane produced around the egg when normally fertilized, and in keeping with this, very few of the eggs segment. It is difficult to say whether or not every egg that cleaved had previously undergone a cortical change. It is difficult even to say if an egg has cleaved or not, for the cleavage patterns have been so variously modified, are so irregular and these are scattered promiscuously among so many eggs that are undergoing degeneration that it is very difficult to determine whether an egg has segmented or is slowly falling to pieces. Many times the cytoplasm of the egg pulls apart, leaving a wedge-shaped opening that finally separates the egg into two approximately equal halves with the nucleus contained entirely within one half; and yet at other times nuclear division may occur without cytoplasmic division. But that there is not a membrane produced that will hold the blastomeres together as in normal fertilization is very evident from the fact that even the first two cells may fall apart and become entirely separate from each other or be held together only within the clear transparent jelly layer of the egg—the chorion layer. But a small percentage of the eggs do produce slight membrane changes: the membrane appears around the egg and can be easily seen with the higher powers of the microscope. They are slightly thickened, clear jelly-like membranes adhering very close to the egg and are not at all to be confused with a normal

membrane. It is therefore possible to assume that all eggs that have segmented have been preceded by cortical changes of some intensity at least.

As one may see from Table I., the largest percentage of cleavages obtained from an exposure to the weaker hypertonic solution (Lot A) was 8 per cent. and that the greatest number of swimming larvæ was 2 per cent., therefore many of the eggs had segmented that did not continue their development to the swimming stage. The optimum effect of the solution for initiation of development was not imparted to the eggs and development ceased after a few cleavages. In the great majority of eggs, however, no evidence of initiatory changes are discernible and one would naturally suspect that they were yet capable of fertilization, and this, as the experiment shows, is partially true. The shorter exposure eggs when inseminated immediately after return to sea-water, react quite normally with sperm, producing a fertilization membrane, normal cleavage and a high percentage of swimming larvæ. But as the exposures are lengthened there is a gradual decline of the normal conditions until an exposure to hypertonic solution, sufficiently prolonged, will entirely prohibit fertilization of the eggs.[1] After an exposure, lasting 25 to 30 minutes, to this concentration, the eggs begin to show a tendency for loss of pigment: they are lighter in color and a few are so affected as to be reduced to mere shadows of their former condition, but this is much more true of the longer exposures.

The character of cleavage and of larval development also declines from the normal as the curve of fertilization approaches zero. In the experiment cited, dishes A 1 and A 2 gave practically normal cleavage and 85 per cent. to 95 per cent. of swimming forms that appeared practically normal. Beginning with dish A 3 however and extending throughout the series of exposures, cleavage became less and less normal; the cleavage planes had grown more indistinct and in many eggs division of the nucleus had gone on while the cytoplasm remained unsegmented. Some of the dishes presented such an indistinct lot of cleavages that it was almost impossible to judge whether or not an egg

[1] Lack of fertilization after exposure to solutions of various salts, has been pointed out by R. S. Lillie, '11.

had cleaved but the cleavage count is as nearly correct as very careful examination could make it. The larvæ appearing in dish A 1, after insemination, were a good lot: approximately 70 per cent. swam at the top of the water and more than 50 per cent. are entirely normal. Compared with the swimming forms of A 5, obtained after fertilization, there is a great difference: only a small percentage of the eggs subjected to the hypertonic solution for one hour (A 5) and inseminated immediately, gave rise to swimming larvæ; and of these only a few could be seen swimming at the top of the water. Almost any form of moving masses of protoplasm may be found among the forms of this dish from a very few apparently normal forms to masses that gave only the least bit of quivering movements; blunt masses, angular forms possessing no semblance of morphological similarity to a normal form, dumb-bell shapes one half of which swims and pulls the other lifeless half around with it, bits of spinning protoplasm that appear somewhat as half, quarter or eighth blastulæ are to be found abundantly. As the length of exposure to hypertonic solution is increased before insemination one encounters a more and more abnormal lot of larvæ; there is not only an increase in the degree of abnormality but the percentage of swimmers is gradually reduced until dish A 11 (inseminated) contained only 10 per cent. of eggs that gave the least signs of life, and of these not any swam at the surface of the water; A 12 (inseminated) revealed not a single form that approached the normal.

It is well to point out again the inability of one to obtain an absolutely correct larval count, for among the eggs that have not been fertilized are many that have undergone cytolysis; the count is only relatively correct but it will be sufficient to illustrate the decrease of the capacity for fertilization of eggs that have been exposed to the activating influence of the weaker solution of hypertonic sea-water.

When the more concentrated solution is used as an activator and is followed by insemination, a different condition is encountered. After the hypertonic treatment alone, of say thirty minutes, one obtains a much larger percentage of cleavage and a decidedly higher percentage of swimming larvæ than is

obtained after the same exposure to the weaker solution. But a comparatively short exposure (5 to 10 min.) to the stronger concentration results in the production of quite a considerable number of membranes. The membranes too are decidedly more pronounced than those produced after exposure to the weaker solution but are not yet like the fertilization membrane produced in normal fertilization, even though the physiological processes responsible for their production may be analogous except perhaps in intensity. Cleavage, after this exposure, although departing very greatly from the normal, is not characterized, as are many of them after the weaker solution, by the separation of the blastomeres; the cells remain in close proximity to each other—a fact that may possibly be associated with the more rigid membrane that is produced around the egg—and a larger percentage of them reach the swimming stage than of those from the other solution. But here also as the length of exposure is increased and the curve of cleavage falls, the same decline in degree of normality and of percentage of swimming larvæ is apparent.

In keeping with the more pronounced effect of the stronger solution in the production of membranes is the fact that the eggs suffer a greater increase in permeability. Loss of pigment is more pronounced than in the case of the weaker solution as one would expect. A small percentage of swimming larvæ obtained after a relatively short exposure to the stronger solution are somewhat normal, but the lots grade so gradually into abnormality and immobility that many can be seen to possess but very little power of movement. Many half, quarter or eighth sized larvæ are found here, as well as following an exposure to the weaker concentration.

There is revealed here a general shake-up of development that is quite characteristic, not merely for the hypertonic treatment, but for development after treatment with any agent that tends to destroy the delicate physico-chemical balance of an egg.

This experiment which may be considered a typical one, offers no indication that the addition of a sperm suspension to eggs that have been activated by hypertonic sea-water are yet

capable of being fertilized, but on the contrary many eggs that have failed to respond outwardly to the activating influences, by segmenting, are found to be incapable of fertilization. The reaction differences of the eggs or their different physiological states assert themselves by the variety of reactions given by the egg after insemination; one egg will segment and produce a swimming larva; another will divide a few times but finally disintegrate; yet still another will remain to all appearances in a normal condition, being normal in size and color, will be surrounded with spermatozoön and yet does not divide; while finally one will retain its full rounded appearance but give up practically all its pigment content to the surrounding water, but does not segment. If the eggs are given the optimum exposure for cleavages,[1] insemination does not increase either the percentage of swimming larvæ or of cleavages.

Professor Loeb, however, makes the claim that sea-urchin eggs may undergo activation, from an exposure to hypertonic sea-water, to such an extent that they segment once or twice, as the case may be, but are nevertheless still capable of fertilization. Quoting from Loeb:[2]

"When we put the unfertilized eggs of *Strongylocentrotus purpuratus* directly into hypertonic sea-water . . . and if we put them back at different intervals into normal sea-water, we find that if eggs have been exposed a sufficiently long time (two hours or more) to the hypertonic sea-water a number will begin to segment. These eggs will often go into the two- or four-cell stage, or sometimes to the eight- or sixteen-cell stage, and then stop developing . . . such eggs remain after this perfectly normal and they have the appearance of small unfertilized eggs. If we wait for sometime, say twenty-four hours, to make sure that they neither develop nor disintegrate, and add sperm, each one of these blastomeres forms a tightly fitting membrane. They begin to develop in a perfectly normal way and into normal larvæ."

Whether or not the fundamental principles of development in the egg of *Arbacia* differ from those of *S. purpuratus*, the

[1] In this particular experiment an exposure to the stronger solution for 30 minutes.

[2] " Art. Par. and Fert., p. 237.

results obtained by the writer with the eggs of *Arbacia* do not agree with the observations of Professor Loeb on the eggs of the California sea-urchin.

The eggs of *Arbacia* will, as does the above cited instance of behavior of the *Stronglyocentrotus* egg, segment into two or four cells and go no farther in development, but remain for several hours apparently normal half or quarter eggs. This condition is perhaps best obtained in *Arbacia* following a hypertonic treatment of thirty to forty minutes, with the weaker solution. If such a lot of eggs are returned to normal sea-water and allowed to remain at room temperature (21°–23° C.) for twenty-four hours, many of them will appear quite normal, while all gradations of loss of pigment can be found. There are present also many small eggs of half or quarter normal size but the majority of these are not normal; most of them are entirely or partially devoid of pigment; some few of them however do appear normal in color. If sperm are added to such dishes and the effects of the insemination carefully noted, a few very striking facts are to be observed:

(1) The smaller eggs as well as the eggs that have not segmented are surrounded by actively swimming spermatozoa but they do not produce a fertilization membrane;[1] (2) many of the unsegmented eggs will segment, but the blastomeres are not held together. Since a good membrane is not produced, the blastomeres may become entirely separated from each other or be retained within the very thin jelly layer of the egg and not be in contact with each other as they are normally. These blastomeres may again divide giving rise, each, to two daughter blastomeres that may again become separate, making four individual blastomeres from the same egg or four quarter-eggs; and these may go on dividing until a great mass of cells are present but not held together. Some few of them (for instance all those derived from one of the first two daughter cells) may remain in loose approximation and perhaps give rise to a very

[1] In many cases eggs that have been exposed to the weaker concentration for a short time only, will produce a quite typical membrane after insemination, but those exposed for one hour to this solution, and allowed to remain standing for twenty-four hours, are almost entirely devoid of any semblance of membrane production after insemination.

abnormal and much smaller swimming larva than the normal. On the other hand, the writer has watched for long periods of time the half- or quarter-eggs found in the dish at the time of insemination but has not found for certain a single one of these that produced a membrane or has segmented as a result of fertilization. But if one should only casually observe the eggs following such a treatment there is no way to distinguish between the separated blastomeres of an egg that has been fertilized and has segmented into two blastomeres that have become separate, and one of the half- or quarter-eggs that was present at the time of insemination. Of importance also is the fact that the *character of a swimming larva of reduced size found in these inseminated cultures is no criterion that would indicate from what kind of an egg it was derived.* As before stated, swimming larvæ of all sizes and of most any external shape assumed by protoplasm can be found in dishes, the eggs of which have been exposed to the hypertonic treatment alone. How then may we conclude that a half- or quarter-sized larva obtained in such a mixed culture after insemination, was derived from a half or quarter-sized egg by fertilization? The only possible way in which the writer could know the exact conditions of the egg from which the swimming larvæ came was by the very laborious method of isolation of individual eggs, and this method was adopted as a last resort.

Four or five hours after an exposure to the weaker concentration of hypertonic sea-water one often finds that 2 per cent. or 3 per cent. of the eggs have segmented once, producing two equal blastomeres that are separated from each other but remain enclosed by the thin transparent jelly layer of the egg. By means of a very fine capillary pipette connected with a flexible rubber tube held in the mouth, these two separate cells, or half-eggs, were isolated from the cultures while in focus under a binocular microscope and collected into a small dish. Thus it was definitely known that only blastomeres of one kind were present. These isolated half-eggs could then be observed and be inseminated in a pure culture, and hundreds of them were isolated and studied in this manner, enabling the writer to determine the following points:

(*a*) Not a single blastomere of the first cleavage of an egg (a half-egg) isolated and left standing in normal sea-water at room temperature, appeared entirely normal at the end of twenty-four hours.

(*b*) A few of these blastomeres, without further treatment, segmented again and some many times. This cleavage was often delayed some hours after treatment, indicating that developmental processes were going on very slowly.

(*c*) Not a single normal larva was obtained out of hundreds of these isolated blastomeres inseminated seven hours or longer after the treatment.

(*d*) These inseminated, apparently normal blastomeres did not form a fertilization membrane.

(*e*) Out of the hundreds of half-eggs that have been inseminated less than 10 per cent. have ever segmented. It is just possible that some of these cleavages have been due to activation by spermatozoa, but the eggs had been so weakened that cleavage was abnormal and never reached the swimming stage. *But beyond doubt most of these cleavages were due to the slow development going on within the egg similar to* (*b*) *above.*

With these facts in mind is it not possible to account for the results of Loeb without considering that his half- or quarter-larvæ came from the fertilization of the separated blastomeres of the 2- and 4-celled stage? As pointed out above, eggs that have remained standing for 24 hours after hypertonic exposure do not produce membranes after insemination, that are rigid enough to hold the blastomeres together; and in these experiments, as well as in Loeb's, many dwarf and badly deformed larvæ can be seen in the culture but they do not come from the fertilization of a half- or quarter-egg, but rather from an egg that was fertilized and later lost cells. Numbers of swimming forms can be seen trailing a comparatively huge mass of cells after it, that have been lost from the egg itself and consequently has resulted in a reduction in size. Even in the cultures producing swimming larvæ from the effects of the hypertonic solution alone, one encounters these half-, quarter-sized, and even smaller swimming masses representing parts of eggs, but these too are reduced in size from loss of cells during the division of a

whole egg and possibly also from an egg, one half of which may have become separated after the first cleavage.

The fact that among these 24-hour cultures of eggs, insemination is not usually followed by a perceptible membrane, may lead one to consider the second division of an egg as a division of two half-eggs that have segmented since insemination but not as a result of it. If these first two blastomeres from the cleavage of an egg are separated and each divides again, how is one to judge whether this is the real condition or that perhaps they may represent the fertilization of a half-egg? Decidedly not by periodical examination, but only by continual observation, if in a mixed culture, or by isolation; and as has just been pointed out, hundreds of isolated blastomeres have been inseminated in pure culture but not a single swimming form has been obtained from these, that have remained at room temperature seven hours between the time of hypertonic treatment and insemination.[1] Whether this explanation will serve to harmonize the differences of opinion or whether there is an individual variation between the fundamental processes of development in the two different species of sea-urchin eggs, the writer is unable to say.

But to obtain a further insight to the observable effects of the addition of sperm to eggs that have been caused to start their developmental processes, eggs from a large number of experiments have been preserved and sectioned and the cytological results are presented briefly in a following section.

2. *Fertilizin and Fertilization.*

In a former paper the writer considered in some detail the relation of the presence of the sperm agglutinating substance, fertilizin, liberated from the normal eggs of arbacia into seawater in which they have been standing, and the capacity of eggs for fertilization, both normal eggs and eggs that had been treated with various reagents. It is not the purpose of the

[1] Some time must be allowed before insemination because of the fact that some of these blastomeres continue to divide before insemination; even seven hours does not entirely eliminate this source of confusion, for a blastomere may divide after that length of time. Developmental changes are still going on, but at a very slow rate.

present paper to take up for discussion the disputed points of the fertilizin theory[1] nor to discuss the way in which fertilizin acts or its rôle in the process of fertilization, but it is perhaps desirable to add another instance of the existing parallelism between the presence of a detectable amount of this agglutinating substance and the capacity for fertilization. Professor Lillie and the writer have previously shown this parallelism to exist in all cases thus far tested: (1) Normal, ripe *Arbacia* eggs liberate this substance into the sea-water as long as they are capable of fertilization; eggs whose germinal vesicles have not broken down do not liberate fertilizin in detectable quantities and they cannot be fertilized. (2) If *Arbacia* eggs have been exposed to the optimum concentration of butyric acid and full membranes were produced, no fertilizin was detectable and the eggs could not be fertilized. (3) If eggs are over-exposed to the same concentration of butyric acid (1 to 3 mins.) the eggs can be, at least, partially fertilized and fertilizin is readily detected. (4) If they are exposed for two hours to this same concentration of butyric acid (50 c.c. sea-water + 2.8 c.c. N/10 butyric acid) they cannot be fertilized and fertilizin is not liberated in detectable quantities. (5) If eggs are exposed to heated sea-water of 35° C. for 10 minutes they could not be fertilized nor could fertilizin be detected; and finally to be added from these experiments. (6) If eggs are exposed to a hypertonic sea-water solution for one to one and one-half hours, sometimes some of the eggs are still fertilizable to a limited extent but always such conditions have been accompanied by a detectable amount of fertilizin; but in conditions where the eggs are not capable of fertilization no fertilizin has ever been detected. Only one experiment will be given in detail to show this parallelism (see Table II.).

From microscopical examination at 5:00 P.M., August 15, one could see that many eggs of the X lot were apparently normal in appearance; they were well rounded, and seemed to possess practically the normal amount of pigment, but upon insemination they did not produce fertilization membranes and the blastomeres after segmentation were not held together, and not one of them developed to the swimming blastula stage.

[1] See Lillie, '14, Moore, '16.

Experiment: Aug. 14, 1916.

2:30 P.M. Eggs collected and washed—divided into lot X and lot Y.

3:05 P.M. X put into (50 c.c. sea-water + 8 c.c., $2\frac{1}{2}$ m. NaCl). Y put into (50 c.c. sea-water + 16 c.c., $2\frac{1}{2}$ m. NaCl).

4:25 P.M. Both lots returned to sea-water and lightly shaken to free from jelly, and series of washings begun.

TABLE II.

Time.	Designation.	Lot X.	Lot Y.
4:50 P.M.	Washing 1	$\frac{10}{1.0}$[1]	$\frac{10}{1.0}$
5:10 "	" 2	"	"
5:50 "	" 3	"	"
6:00 "	Inseminated	Cleavage 10 per cent.	0
Aug. 15			
8:30 A.M.	Washing 4	$\frac{10}{1.0}$	$\frac{10}{1.0}$
9:10 "	" 5	"	"
10:45 "	" 6	"	"
11:10 "	" 7	"	"
12:30 P.M.	" 8	"	"
2:10 "	Tested for fertilizin	8 second reaction	negative
	Inseminated	Cleavage 13 per cent.	0
2:15 "	Washing 9	$\frac{10}{1.0}$	$\frac{10}{1.0}$
3:00 "	" 10	"	"
3:20 "	" 11	"	"
4:00 "	Test for fertilizin	5 second reaction	negative
4:20 "	Washing 12	$\frac{5.0}{1.0}$	5.0 1.0
5:00 "	Tested	4 second reaction	negative
	Heavy insemination	Cleavage 20 per cent. (poor)[2]	0
5:20 "	Eggs killed in Boveri's picro-acetic acid		

The results of this experiment have been confirmed by a number of others and the results have always been consistent—if the supernatant fluid possessed enough fertilizin to cause the agglutination of a fresh sperm suspension, always there had been eggs present in the culture that possessed enough latent developmental capacity to react with a spermatozoön and to segment as a result of this union, but if no fertilizin could be detected never have the eggs been found to possess a capacity for fertilization. Some change in the physiological or physico-

[1] In every case the numerator of the fraction represents the volume of eggs and sea-water while the denominator represents the volume of eggs and sea-water (in c.c.) remaining in the tube after most of the supernatant sea-water had been drawn off—for further details see footnote 1 on page 276.

[2] No larvæ produced from 5 P.M. insemination, in either lot X or lot Y.

chemical state of the egg has been brought about as a result of the hypertonic treatment and fertilization is impossible. Of what this change consists we do not know, but it is at least accompanied by an increased permeability of the egg and loss of substances from it. Whether a specific substance necessary for fertilization has diffused from the egg or whether it has undergone new chemical combinations that have rendered it unavailable and whether it may be the substance called fertilizin are questions that cannot now be answered. But it seems significant that here as in all previously modified conditions there has always been a substance escaping from the egg into the surrounding sea-water that could be detected by its sperm agglutinating properties, if the eggs were still capable of fertilization. That this unfertilizable condition is due to a new physiological state of the egg itself and not to a physical condition of the surface of the egg that might prevent sperm entrance, is made certain by observation on preserved material. Sperm do penetrate these eggs and sometimes produce weak changes similar to fertilization or they remain entirely ineffective, depending upon the condition of the egg itself.

3. *Conclusions of Section III.*

The results of this series of experiments seem to indicate that a certain optimum exposure of the eggs of *Arbacia* to hypertonic sea-water initiates changes within the egg that result in development. That all do not segment is because the optimum exposure for one egg is not the optimum for all.[1] Some have been over-exposed, some under-exposed, but if the exposure was the optimum one, swimming larvæ were produced; if less or greater than the optimum perhaps only one or two cleavages resulted and development ceased; if decidedly over-exposed or under-exposed, cleavage does not occur. This quantitative relation is then interesting from the standpoint of fertilization. If the eggs were not perceptibly affected by the exposure they could be fertilized; if the reactions initiated by the exposure were of sufficient intensity to disturb the physico-chemical condition of

[1] The difference may be due to the factor of aging as Goldfarb ('16) has applied the term.

the egg and cause the loss of substances necessary for fertilization or has caused new chemical combinations to be formed that renders certain substances unavailable, then fertilization is likewise impossible.

In order to determine definitely whether or not this unfertilizable character of the egg is due to a real physiological or physico-chemical condition of the egg substances or whether it is due to physical conditions that prevent the extrance of a spermatozoön into an egg, a large number of these from different kinds of experiments were preserved and sectioned. Usually Boveri's picro-acetic acid was used as a killer and sections (4 μ thick) were stained in iron hæmatoxylin.

IV. Observations from Preserved Material.

The cytological data to be presented is not meant to be a critical analysis of chromosome behavior but is given only to acquaint us with a few of the more general facts of the behavior of sperm in relation to these unfertilizable conditions, and it will be confined to observations upon three different classes of experiments: (1) To mass cultures of eggs that have been exposed to the weaker concentration of hypertonic sea-water for different lengths of time, returned to normal sea-water and immediately inseminated; (2) to mass cultures which have been exposed to the stronger concentration of sea-water for different lengths of time, returned to sea-water and inseminated; (3) to pure cultures of eggs that have segmented but once after an exposure to the weaker concentration, and inseminated.

1. *Insemination Following an Exposure to the Weaker Hypertonic Solution.*

Experiment:

2 P.M. Eggs were collected, washed three times.

2:50 P.M. Transferred to hypertonic sea-water—(50 c.c. sea-water + 8 c.c., $2\frac{1}{2}$ m. NaCl).

Eggs returned at intervals to sea-water, divided into lots A and Ax. Lot A no further treatment; control.

Lot Ax, immediately inseminated after return to sea-water. Eggs from both lots killed as given in Table III.

Table III.

No.	Lot A.			Lot Ax.			
	Returned to Sea-water.	Per Cent. Cleavages.	Per Cent. Swimming Larvæ.	Per Cent. Cleavages.	Per Cent. Larvæ.	Insem-inated.	Killed.
1	3:10 P.M.	2	1	85	75	3:15 P.M.	3:45 P.M.
2	3:30 "	2	0	75	75	3:35 "	4:05 "
3	3:50 "	15	1	45	15	3:55 "	4:25 "
4	4:10 "	25	3	30	8	4:15 "	4:45 "
5	4:30 "	12	0	12	1	4:35 "	5:05 "
6	4:55 "	20	1	12	2	4:55 "	5:25 "

Microscopical Observations on Lot A.

3:30 P.M., A^1 eggs appear normal.

3:55 P.M., A^2 eggs appear normal.

4:15 P.M., A^3 eggs appear normal.

4:40 P.M., A^4 10 per cent. eggs show loss of pigment.

5:10 P.M., A^5 few eggs almost colorless; 30 per cent. appear normal.

5:20 P.M., A^6 some eggs reduced to shadows, many appear quite normal.

Sections of eggs of the AX^1 lot appear not greatly different from lots that have been normally fertilized; very few of them

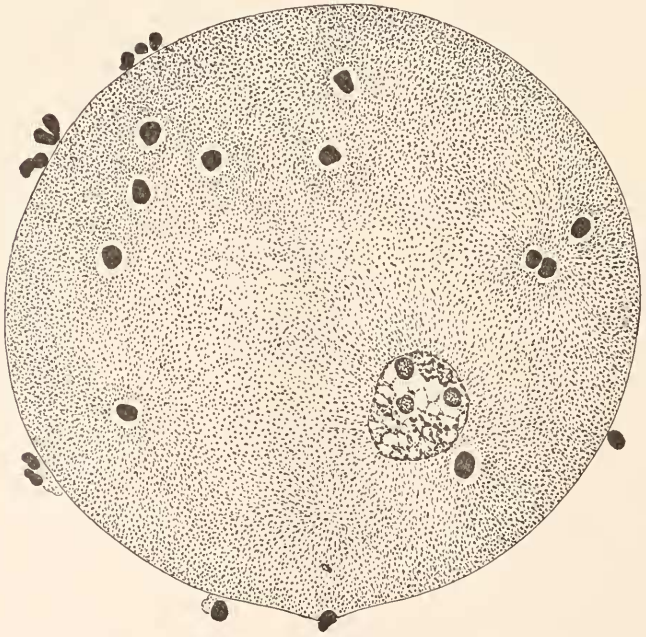

Fig. 2. × 3,000. Section of an egg exposed to the stronger hypertonic solution for 15 minutes and inseminated immediately on return to sea-water. Killed 15 minutes after insemination. The sperm heads are entirely unchanged and have not reacted with the egg.

show more than one sperm nucleus and this has usually undergone the characteristic swelling and vacuolization changes of a male pronucleus and is accompanied by an aster. After a longer exposure to this concentration of hypertonic sea-water the resemblance to sections of normal fertilization gradually disappears and is superseded by a general condition of polyspermy and later by a non-reactive state of the egg substances,

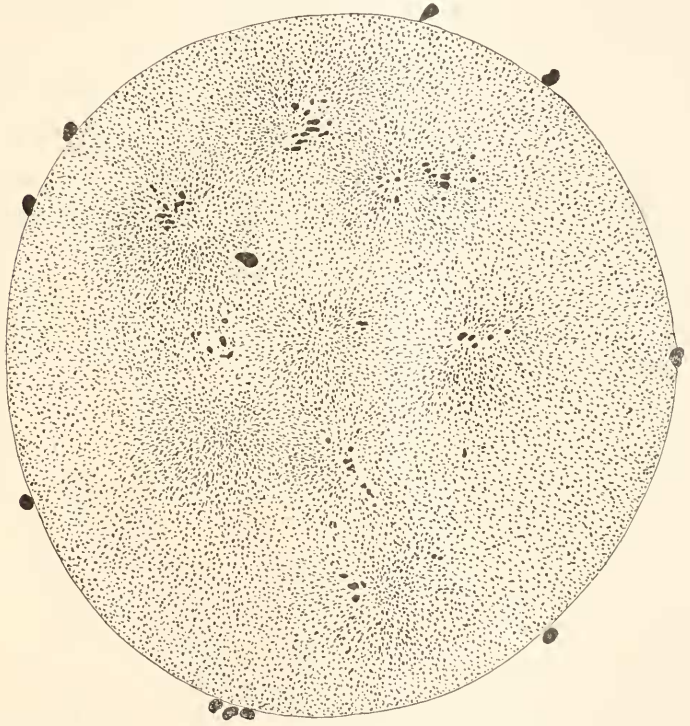

FIG. 3. × 3,000. Section of an egg exposed to the weaker hypertonic solution for two hours and inseminated immediately on return to sea-water. Killed 20 minutes after insemination. All but one of the sperm heads are undergoing fragmentation. The egg nucleus does not appear in this section.

with an abundance of spermatozoa, lying within the cytoplasm, entirely unchanged. Of the eggs of AX^3, probably the larger percentage are polyspermic and show a reactive condition of the sperm heads; they appear swollen and slightly vacuolated and are usually accompanied by an aster more or less well formed. Some sections of this lot still appear almost normal in their

general characteristics. In lot AX^5 however some of the sperm heads are slightly swollen but are not associated with astral radiations and still others are surrounded with weak radiations. Many cases are encountered in which the egg nucleus has broken down into chromosomes, dispersed along the rays of a unipolar spindle, formed by the egg, as a result of the hypertonic treatment; and at the same time several sperm nuclei may lie unchanged within the cytoplasm of the egg.[1] In AX^6 most of the eggs have almost completely lost their capacity to react with a spermatozoön. The majority of the sections show the sperm heads entirely lacking an aster, but many asters produced by the effects of the hypertonic solution are present and are very definite. The reaction between the egg and sperm is largely negative. In numerous sections the physiological changes within the egg cytoplasm have not only prevented changes characteristic of normal fertilization, but they are even of such a character as to cause the disintegration of sperm heads. Fig. 3 is a section of such an egg and shows one sperm head yet unchanged but several others are undergoing dissolution. Other sections show another type of disintegration which is characterized by a swollen vesicle about the sperm head which is breaking apart and stains very faintly with iron-hæmatoxylin.

2. *Insemination Subsequent to an Exposure to the Stronger Solutions.*

The examination of sections of eggs of the following experiment serves to give a general understanding of the incapacity for fertilization, after eggs have been exposed to the stronger solution of hypertonic sea-water.

Experiment:

4:30 P.M. Eggs collected, washed three times.

5:10 P.M. Transferred to hypertonic sea-water—(50 c.c. sea-water + 16 c.c., $2\frac{1}{2}$ m. NaCl).

Eggs transferred at intervals to sea-water and divided into two lots X and Y. Lot X remains standing without further treatment. Lot Y inseminated immediately after return to sea-water. Eggs were removed from each lot at definite times and preserved as given in Table IV.

[1] See Wilson, '01, and Hindle, '10.

TABLE IV.

No.	Returned to Sea-water	Lot X. Percentage of		Lot Y. Percentage of	
		Cleavages	Swimming Larvæ.	Cleavages.	Swimming Larvæ.
I	5:15 P.M.	10	0	25	3
II	5:20 "	6	0	12	7
III	5:25 "	5	2	12	3
IV	6:00 "	20	2	22	2

Series Preserved in Picro-acetic Acid.

I x killed 5:30 P.M.	I y^1 killed 5:30 P.M.	I y^2 killed 5:45 P.M.
II x " 5:50 "	II y^1 " 5:35 "	II y^2 " 5:50 "
III x " 5:25 "	III y^1 " 5:40 "	III y^2 " 5:55 "
IV x " 6:15 "	IV y^1 " 6:15 "	IV y^2

The general cytological observations on this preserved series are as follows: In all lots of eggs exposed to the stronger concentration, from five minutes to fifty minutes, and inseminated immediately upon their return to sea-water, sperm have penetrated the eggs freely and lie in any plane of the egg cytoplasm from the periphery to the center. No attempt has been made to determine by actual count, from sections, the relative proportion of eggs containing spermatozoa, but this condition is found under every field of the microscope and many sections of eggs show from two to sometimes twenty sperm heads. By far the larger number of eggs show the presence of sperm heads imbedded within the cytoplasm.

The effects of the egg cytoplasm environment upon the individual spermatozoön varies in the different eggs in which they are found. Many sections prove by their presence that three, four or more spermatozoa have entered the egg and react with it, thus giving the picture of a typical case of polyspermy; the sperm heads may be separated from the egg nucleus and accompanied by a sperm aster, or two or three may have copulated with the egg nucleus. In other sections a dozen spermatozoa may be scattered throughout the cytoplasm, but thirty minutes after insemination they exhibit not the slightest perceptible change in size or shape (see Fig. 2). They appear perfectly solid with no indications of vacuolization; there is no indication of a sperm aster, and in short the sperm heads appear only as inert foreign

bodies in the cytoplasm of the egg.[1] Still other sections exhibit a different type of reaction of the sperm to its new environment, that appears significant—the sperm heads may undergo swelling and vacuolization without any indication of the appearance of a sperm aster. For the most part asters arise only in connection with a spermatozoön that shortly appears to be in the swollen, rounded, and slightly vacuolated condition but, as has been mentioned, some possess much more pronounced astral rays than others. This condition of the entire absence of a sperm-aster from the spermatozoön undergoing the more or less characteristic changes of activity, probably to be referred to chemical influences exerted upon it by some substance within the egg, seems to indicate a quantitative reaction of the spermatozoön with the egg, the sperm aster formation being the more sensitive factor. In fact this series of preparations exhibits a gradient of the effects of a spermatozoön, from essentially its normal effect, to a condition in which it provokes not the slightest change; and since a sperm aster is usually associated with a swollen sperm head cannot this be an indication of the suppression of sperm aster formation but a continuation of the more stable changes accompanying sperm activity?

(*a*) *Penetration of the Spermatozoön.*—An interesting condition for the observation of sperm entrance is presented by this series of preparations and since the writer knows of no observations on sperm entrance in *Arbacia* some passing mention of it may be of interest. As may be seen from Fig. 4, two spermatozoa are in the act of entering an egg and many other such sections are to be found among the series. The egg has produced a small protrusion of protoplasm as an entrance cone that projects slightly above the egg surface; and within this cone lies the sperm head pulled out into a narrow chromatin band, one end of which projects down into the cytoplasm. No definitely formed fixation body, to which the inner end of the spermatozoön is attached, has been observed but the very shape and character of the entering spermatozoön would indicate a decided attraction from within the egg that acts upon the sperm head.

[1] Normal sperm asters appear within five to ten minutes. Around most of the sperm heads is a small clear area produced by the retreat of protoplasmic granules of the egg from its periphery.

Fig. 4 was made from lot III Y[1] (page 283) and perhaps cannot be considered as representing the normal mode of entrance of the spermatozoön of *Arbacia* but it at least indicates the possible method of entrance. It may be necessary to attribute the appearance of the phenomenon to the general retarded condition of the egg protoplasm brought on as a result of the hypertonic treatment before insemination, but it would not seem impossible to discover a somewhat similar condition of sperm

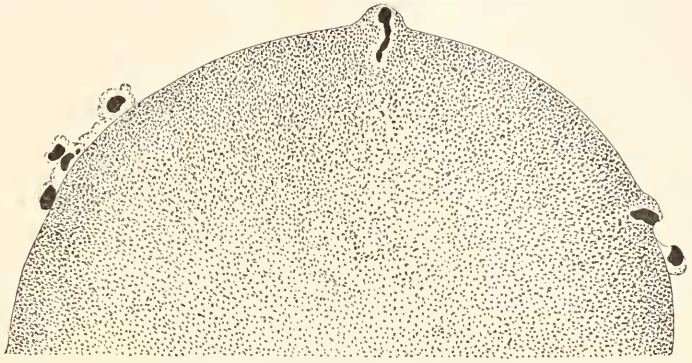

Fig. 4. × 3,000. Part of a section of an egg showing the entrance of the spermatozoön; one has practically completed its entrance and is rounding up, the other in the process of entrance. From same lot as Fig. 2.

activity under normal conditions if the right stages were preserved in a close enough series. Penetration, in *Arbacia*, is effected much more readily than in *Nereis*, in which Professor Lillie[1] has illustrated so clearly the entrance of the spermatozoön. The most essential difference in the morphology of the process in the two forms is the absence of the attraction cone in *Arbacia* which in *Nereis* forms an attachment for the spermatozoön and apparently pulls the spermatozoön through a small aperture in the vitelline membrane as it recedes from the periphery of the egg. Immediately after the spermatozoön is taken inside the egg it assumes its normal appearance, for no elongated heads have been observed that were not in the act of entering the egg.

3. *Insemination of Pure Cultures of Isolated Blastomeres.*

Since the experimental results of the writer did not agree with the views of Loeb—namely, that eggs whose development

[2] See Lillie, F. R., '11 and '12.

had been initiated by an exposure to hypertonic sea-water, could yet be fertilized, produce fertilization membranes and develop into swimming larvæ, he was anxious to know if fertilization was prevented by failure of the sperm to enter or whether the individual blastomeres—the products of the first cleavage of an egg—had developed an immunity to the latent effects of a spermatozoön.

It is obvious that to determine this point from cytological preparations a pure culture of the first two blastomeres was highly desirable; and with considerable difficulty some two hundred and fifty or three hundred eggs that had segmented but once were isolated from lots of eggs that had been subjected to the weaker hypertonic solution. This pure culture of approxi-

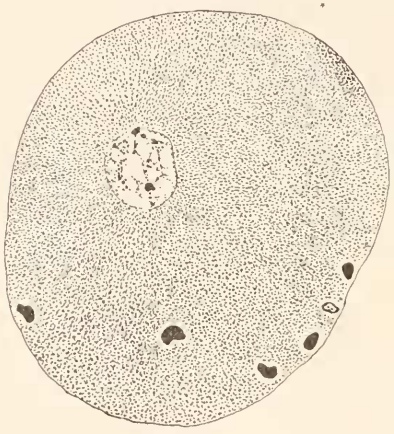

FIG. 5. × 3,000. Section of a quarter egg showing the presence of sperm nuclei. Weaker hypertonic solution, 30 to 40 minutes; egg segmented once, isolated, each blastomere segmented again. Killed 20 minutes after insemination.

mately five hundred half-eggs was heavily inseminated, allowed to stand for twenty minutes and preserved in Boveri's picro-acetic acid. A very few of these blastomeres had divided again into two daughter blastomeres (quarter-eggs) before the lot was inseminated, but every egg was isolated from the mass culture into a pure culture while in the two-celled stage.[1]

Microscopical sections of these half-eggs prove to us beyond

[1] As a definite membrane was not produced by the egg following the hypertonic treatment the blastomeres became entirely separated from each other in the pure culture. All were half-eggs when isolated.

any possible doubt that sperm do enter the blastomeres (Fig. 5) but the normal effect of a spermatozoön entering an egg is not apparent. Many of the sperm heads lie within the cytoplasm entirely unchanged, while others are seen to have increased in size from swelling, but most of these are undergoing evident degeneration, of which various stages can be seen (Fig. 6). The chromatin gradually loses its staining reaction, appearing much clearer than the unchanged sperm heads outside of the eggs, and some have so entirely lost their staining capacity that they appear only as clear vacuoles scattered throughout the cytoplasm of the half-eggs. Out of the vast numbers of sperm heads observed within these half-eggs only one was found that contained an indication of a good sperm aster. A few of the

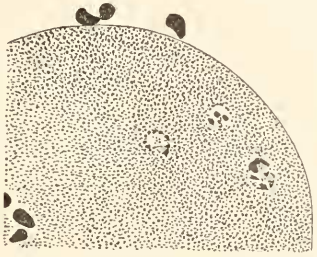

FIG. 6. × 3,000. Part of section of a quarter egg showing presence of both unchanged and degenerating sperm nuclei. No indication of fertilization changes. Same exposure as Fig. 5.

blastomeres are undergoing segmentation as a result of the hypertonic treatment and the spindles appear quite normal.

Here then the spermatozoön not only does not find a congenial environment in which normal reactions can go on, but the surroundings are even hostile and result in the disintegration of the spermatozoön without its having undergone any changes that indicate the least possibility of a fertilization reaction.

4. *Conclusions of Section IV.*

This cytological examination very clearly reveals the fact that the unfertilizable condition of eggs subsequent to an exposure to hypertonic sea-water is not due to the failure of a spermatozoön to enter the egg but that it is due to a change within the egg itself. Certain changes that are to be associated

with initiation of development have produced an environment in which the reaction between a spermatozoön and an egg has been rendered impossible. *Spermatozoa enter the eggs in comparatively large numbers but the eggs are not fertilized.*

In the shorter exposure to hypertonic sea-water many eggs do react with a spermatozoön but since these are conditions in which a very small percentage of eggs segment and a still smaller percentage reach the swimming stage after the hypertonic treatment, the observations are interpreted as indicating an effect of the hypertonic solution below the optimum exposure for the initiation of development and as a consequence the eggs have experienced but little change from the normal condition; the exposure did not lead to the initiation of development, therefore the eggs still possess their capacity for fertilization. That this lack of a capacity for fertilization is one that is gradually developed as the exposure to the hypertonic solution is increased, is one of the factors that indicates the quantitative side of fertilization. After a short exposure, polyspermy, in which all sperm seem to have a part, is the general rule; a more pronounced effect of the hypertonic sea-water results in a greater number of sperm entering the egg, some showing very weak asters, others being only slightly swollen, while yet a more decided influence may create an environment within which the spermatozoön is not only inactive but also it may even undergo disintegration. Whatever changes have resulted from treatment with hypertonic sea-water, spermatozoa that gain entrance to eggs that have been exposed to optimum conditions for segmentation, or that have been decidedly over-exposed, do not meet with an environment that will permit of a reactivation of the egg.

This degeneration of spermatozoa in a non-reactive egg environment calls to mind essentially similar conditions, encountered normally, in the fertilization of meroblastic eggs such as the pigeon egg described by Blount ('07). Polyspermy in meroblastic eggs appears to be normal but the accessory spermatozoa, after the environment has been changed by reactions within the egg set up by a reaction of the egg with the successful spermatozoön, are repelled peripherally and degenerate and disappear.

V. Discussion.

For an egg to develop, some stimulus must initiate certain changes within it which, having been once set in motion, appear to be autonomous. If an egg is not fertilized, or its development initiated by some artificial agent, it normally does not develop. But studies in the field of the artificial initiation of development have brought to light many methods that may be employed successfully to set in motion the developmental mechanics latent within the egg. As yet we are unable to determine just the mechanism by which these agents are effective, but practically all methods agree in one essential at least—the permeability of the egg membrane is increased.

Practically every one is agreed that the egg must be considered, a very delicately adjusted physico-chemical system, within which, if a spermatozoön gains entrance, a characteristic series of reactions are set in motion that lead to development and a consequent change of the system. If artificial agents are employed as a substitute for the spermatozoön not only do we have conditions changed but also if the proper procedure is known and applied the end results are essentially the same as when initiation was effected by a spermatozoön, *e. g.*, cleavage, gastrulation and swimming larvæ. After the initiatory changes have been induced a spermatozoön in either case is entirely unable to produce any of its characteristic effects.

The delicately adjusted condition of the egg is only a temporary thing for if sea-urchin eggs are allowed to stand in sea-water for a day or more they gradually lose their power of fertilization. Not only can this unfertilizable condition, accompanied by increased permeability, be produced by standing but also by hypertonic sea-water, butyric acid, by a slight rise in temperature of the sea-water and several other agents including, of course, fertilization.

Many writers have considered an unfertilizable condition as an indication of the death of the egg but to cite only one instance to disprove this, the writer has called attention to the fact that if initiation of development is partially accomplished by a treatment with butyric acid and the membranes so formed are destroyed by shaking, sperm will enter the eggs of *Arbacia* but do

not assert any capacity for the initiation of development: however if the eggs were subsequently exposed to the proper treatment with hypertonic sea-water a goodly per cent. will develop to swimming plutei. The non-fertilizable condition is not due to the death of the egg but to a changed physiological condition induced by the initiatory action of the butyric acid treatment. Whatever the changes brought about, all of the conditions mentioned above are accompanied by the loss of substances from the egg, if indeed the condition is not a result of the loss of some substance or substances that are necessary for fertilization. This loss may be due either to the escape from the egg of the necessary substance or to new chemical combinations within the egg made possible by the disturbance of its physiological equilibrium.

It is highly interesting to note that a qualitative and possibly a quantitative test for one substance at least has been discovered by F. R. Lillie. This substance fertilizin is continually liberated from the egg into the surrounding sea-water as long as it remains in a fertilizable condition and as pointed out on page 276 it has been found to afford an absolute test (using a fresh sperm suspension as an indicator) of the capacity of sea-urchin eggs for fertilization in seven analyzed cases and will probably be found to hold for all cases in *Arbacia*, as it is evidently an indicator of a generalized condition.

An analogous condition to that in *Arbacia*, of the loss of substances from the egg and the consequent lack of capacity for fertilization, has been found in other forms than the sea-urchin, though in no other forms than *Arbacia* and *Nereis* has a qualitative test for a substance been found. E. E. Just ('15) has discovered that if eggs from *Platynereis* are collected in sea-water they cannot be fertilized. This is due to some effect of the sea-water in causing the loss of necessary substances for fertilization either through diffusion from the egg or through new chemical combinations within the egg. If only a few drops of sea-water come in contact with the egg they will not fertilize despite the fact that sperm penetrate the eggs and produce weak changes characteristic of fertilization: the proper egg substances for the intiatory reactions are not available.

Miss Allyn ('12) found that any substance that had a tendency to initiate development in *Chætopterus* was detrimental to fertilization. She noted that the tendency was not for the exclusion of spermatozoa but rather for the production of polyspermy. The aberrant type of cleavage became more accentuated as the exposure to the initiatory agent was prolonged and would probably have entirely prevented fertilization with a slightly longer exposure. In this case the initiatory agents induced only a partial completeness of the reactions and as time went on fertilization became less and less possible, indicating the gradual loss of some substance whose presence is indispensable for fertilization. *Arbacia* eggs behave in essentially the same manner if over-exposed to butyric acid; polyspermy appears if insemination is carried out shortly after the exposure, but if the eggs remain standing in sea-water for a few hours insemination has no effect.

Just how hypertonic sea-water is effective we are not able to say, but the writer is inclined to the views of R. S. Lillie that a properly timed exposure to any substance that lowers permeability (unless it be decidedly toxic) allows of the combination of the substances within the egg that starts off the developmental reactions. If these changes are nearly enough the normal, development is quite complete up to certain stages in the life of the organism. The question, however, to which these experiments relate is the capacity for fertilization after activation by hypertonic sea-water.

The quantitative reaction idea has been pointed out quite freely in this and a preceding paper in considering the initiation of development, and certain instances of all phases of this process are to be had not only from the experiments themselves but also from the preserved material. From the purely parthenogenetic standpoint the ideas of the qualitative specificity of hypertonic sea-water as a factor in the initiation of development have been dealt a death blow by the experiments of R. S. Lillie ('15) on starfish eggs, and the purely quantitative idea substituted for the explanation. Lillie finds that not only can starfish development be initiated by a preliminary treatment with butyric acid followed by hypertonic sea-water, but also that a secondary

treatment with butyric acid or warm sea-water or cyanide sea-water may be substituted for the hypertonic sea-water, or that both the preliminary and secondary treatments with warm sea-water may accomplish the same results. From inference any agent or combination of agents that cause a quantitative fulfillment of the reactions lead to the same end.

When a spermatozoön fertilizes an egg the optimum stimulation has been given to the egg, and we assume that the initiatory reactions have been quantitatively complete. But certainly in the initiation by artificial agents some of the eggs will not have been given the optimum conditions and will not have completed their reactions. If we can think in terms of chemical substances we will suppose that part of these have been left uncombined that are yet capable of some reaction with a spermatozoön if it but gain entrance to the egg. Consequently just so far as these fundamental reactions have been incomplete the spermatozoön is capable of asserting its latent stimulus for the activation processes. That fertilization is not complete in such cases is shown by the very irregular type of cleavage and the small per cent. of swimming larvæ and the poor quality of these, just as in a parthenogenetic treatment the same results are encountered if an exposure above or below the optimum one is given. If however the initiatory agent employed, has been affective enough to cause the production of good membranes (butyric acid), or has resulted in segmentation of the egg, the initiatory, reactive, egg substances appear to have been pretty well used up and so far as these experiments show there seems to be no tendency for a regeneration on the part of an egg of the fertilizable condition. The blastomeres of an egg appear to have no capacity, or at the most very little, to react with a spermatozoön. It has been pointed out above that out of hundreds of these isolated blastomeres never has one been able to produce a swimming larva, as a result of fertilization; and sections have revealed but one absolute case of sperm aster formation after penetration. One activation seems to preclude all others.

Fertilization appears to be due to an agent, spermatozoön, that initiates development by producing a condition that permits of the interaction of certain substances within the egg,

which union curtails any further possibility of a subsequent fertilization. If the same conditions are produced by artificial parthenogenesis the same lack of the capacity for fertilization is evident.

Whether the lack of this capacity is due to the absence of one substance or of several substances, and whether the substance called fertilizin is the essential one or is only an indication of a certain physiological state of the egg is unknown. But in every case, so far examined, fertilizin has been present if the eggs could be fertilized and has been absent if there was no capacity for fertilization.

These results are weighty argument, if not definite proofs, against any idea of a sperm-borne substance that is necessary for the initiation of and the continuation of development. There is no evidence that the spermatozoön imparts a "lysin-like" substance that produces cytolysis within the egg and later carries into the egg a "corrective agent" to check the imposed cytolysis. The egg possesses all the substances it needs for development.

VI. Summary.

1. If *Arbacia* eggs are exposed to a weaker and a stronger concentration of sea-water for varying lengths of time a perceptible gradient of effectiveness from the entire absence of any reaction to an essentially normal parthenogenetic reaction is apparent.

2. The effective exposure is accompanied by a perceptible condition of increased permeability and substances escape from the eggs.

3. The superposition of insemination on the optimum hypertonic treatment does not increase the percentage of development.

4. Eggs when exposed to hypertonic sea-water for two hours usually cannot be fertilized; and in such a condition fertilizin cannot be detected by any known method. If however there yet remains a certain capacity for fertilization, fertilizin can be detected by its sperm agglutinating properties.

5. If *Arbacia* eggs are exposed to activating agents the permeability of the egg is increased, substances are lost from it and fertilization is impossible.

6. If eggs are sectioned, after insemination subsequent to an exposure to the weaker hypertonic solution, a gradient of activity of spermatozoa parallels, to a certain extent, the length of the exposure to hypertonic sea-water.

7. If eggs are sectioned after insemination, subsequent to an exposure to the stronger hypertonic solution, large numbers of eggs are seen to have several spermatozoa within their boundaries; but they usually do not exhibit any signs of activity; many are undergoing degeneration.

8. Sperm, in gaining extrance to an egg, are pulled out into a long thin chromatin band situated in the protoplasmic cone produced by the protoplasm of the egg.

9. If a pure culture of the first two cells, produced by cleavage, are inseminated, sperm enter, remain entirely quiescent, and may even undergo disintegration. They do not fertilize the blastomeres.

10. These experiments furnish no evidence for, but are contrary to, any view that a spermatozoön carries a substance into the egg that is necessary for development. There is no evidence of a sperm-borne "lysin-like" substance nor a secondary "corrective agent." The egg possesses all the essentials for development.

HULL ZOÖLOGICAL LABORATORIES,
March 28, 1917.

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